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(54) Title: DIAGNOSIS AND TREATMENT OF METASTIC BREAST CARCINOMA


(57) Abstract

The invention provides a method of detecting an increased likelihood of metastic breast cancer by detecting the levels of $\alpha 6\beta 1$ integrin. The invention also provides methods for the prophylaxis or treatment of metastic breast cancer by blocking $\alpha 6\beta 1$ function.

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# DIAGNOSIS AND TREATMENT OF METASTIC BREAST CARCINOMA

The field of the invention is cancer diagnosis and therapy.

## Summary of the Invention

We have discovered that α6β1 integrin plays an essential role in breast cancer metastisis and that methods of blocking α6β1 function block the metastatic process. The presence of α6β1 allows metastis; blocking of α6β1 function prevents metastisis. Accordingly, the invention provides methods of predicting the likelihood of metastic breast carcinom and methods for predicting the prognosis for patients diagnosed with breast carcinoma. The invention also provides methods for preventing and treating breast carcinoma by blocking α6β1 function.

In general, the invention features a method of diagnosing a mammal for the presence of a metastatic breast cancer or an increased likelihood of developing metastatic breast cancer. The method involves measuring α6β1 integrin in a breast tissue sample from the mammal, with an increase in α6β1 levels relative to a sample from an unaffected individual being an indication that the mammal has a malignancy or has an increased likelihood of developing a malignancy.

In preferred embodiments, the increase in α6β1 integrin levels is two-fold or greater.

Kits for carrying out the above methods are also included in the invention. Such kits preferably include a substantially pure antibody that specifically recognizes and binds a α6β1 polypeptide, and may also include means for detecting and quantitating antibody binding. Alternatively, the kit may include all or a fragment of a α6β1 nucleic acid sequence useful for

hybridization purposes, and may also include means for detecting and quantitating α6β1 RNA hybridization.

The invention also features a method of treating a mammal with a α6β1-associated malignancy involving administering to the mammal a α6β1 antibody in an amount sufficient to inhibit growth of the malignancy, and further features a therapeutic composition having as an active ingredient a α6β1 antibody, formulated in a physiologically-acceptable carrier. Preferably, the antibody is directed to the extracellular domain of α6β1 integrin.

In two other related aspects, the invention features methods of identifying α6β1 inhibitory compounds. The first method involves the identification of inhibitory compounds that are capable of decreasing the expression of a α6β1 gene, involving (a) providing a cell expressing the α6β1 gene; and (b) contacting the cell with a candidate compound, an increase in α6β1 expression following contact with the candidate compound identifying a modulatory compound. The second method involves the identification of modulatory compounds which are capable of decreasing α6β1 function, involving (a) providing a cell expressing the α6β1 protease; and (b) contacting the cell with a candidate compound, an decrease in α6β1 activity following contact with the candidate compound identifying a modulatory compound.

In a related aspect, the invention features a method of treating a mammal with a disease involving increased presence of a α6β1, involving administering to the patient a modulatory compound (for example, identified according to the above methods) in an amount effective to reduce the symptoms of the disease in the mammal.

By "α6β1 polypeptide" is meant an amino acid sequence which is an integrin laminim receptor as

described, for example, in Mercurio, Trends Cell Biol. 5:419-423, 1993, whose expression correlates with the presence of malignant breast carcinoma. Preferably, such a polypeptide has an amino acid sequence which is at least 45%, preferably 60%, and most preferably 85% or even 95% identical to the amino acid sequence referenced in Mercurio (1993), supra.

By a "substantially identical" polypeptide sequence is meant an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the function of the polypeptide (assayed, e.g., as described herein).

Preferably, such a sequence is at least 85%, more preferably 90%, and most preferably 95% identical at the amino acid level to the sequence of Fig. 10 (SEQ ID NO: 1). For polypeptides, the length of comparison sequences will generally be at least 15 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably at least 35 amino acids.

Homology is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, substitutions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic

acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By "protein" or "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

By "substantially pure" is meant a preparation which is at least 60% by weight (dry weight) the compound of interest, e.g., the $\alpha 6\beta 1$ polypeptide or $\alpha 6\beta 1$-specific antibody. Preferably the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

By "purified DNA" is meant DNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5′ end and one on the 3′ end) in the naturally-occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By a "substantially identical" nucleic acid is meant a nucleic acid sequence which encodes a polypeptide differing only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative

substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the function of the polypeptide (assayed, e.g., as described herein). Preferably, the encoded sequence is at least 45%, more preferably 60%, and most preferably 85% identical at the amino acid level to the sequence of Fig. 10 (SEQ ID NO: 1). If nucleic acid sequences are compared a "substantially identical" nucleic acid sequence is one which is at least 85%, more preferably 90%, and most preferably 95% identical to the sequence of Fig. 11 (SEQ ID NO: 2). The length of nucleic acid sequence comparison will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides. Again, homology is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

By "purified antibody" is meant antibody which is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, antibody.

By "specifically binds" is meant an antibody which recognizes and binds a $\alpha 6\beta 1$ polypeptide but which does not substantially recognize and bind other molecules in a sample (e.g., a biological sample) which naturally includes $\alpha 6\beta 1$ polypeptide. An antibody which "specifically binds" $\alpha 6\beta 1$ is sufficient to detect a $\alpha 6\beta 1$ protein product in such a biological sample using one or more of the standard immunological techniques available to those in the art (for example, Western blotting or immunoprecipitation).

By "malignancy" is meant any abnormal tissue that grows by cellular proliferation more rapidly than normal or that continues to grow after growth stimuli cease. Most malignancies show partial or complete lack of structural organization or functional coordination with surrounding normal tissue. A malignancy according to the invention is generally either locally invasive or metastatic.

By "relative to a wild-type sample" is meant either (a) relative to an equivalent tissue sample from an unaffected individual or (b) relative to an unaffected sample of similar tissue type from the mammal being diagnosed.

By "immunological methods" is meant any assay involving antibody-based detection techniques including, without limitation, Western blotting, immunoprecipitation, and direct and competitive ELISA and RIA techniques.

By "means for detecting" is meant any one or a series of components that sufficiently indicate a detection event of interest. Such means involve at least one label that may be assayed or observed, including, without limitation, radioactive, fluorescent, and chemiluminescent labels.

By "$\alpha 6\beta 1$ RNA" is meant messenger RNA transcribed from a $\alpha 6\beta 1$ DNA sequence.

By "hybridization techniques" is meant any detection assay involving specific interactions (based on complementarity) between nucleic acid strands, including DNA-DNA, RNA-RNA, and DNA-RNA interactions. Such hybridization techniques may, if desired, include a PCR amplification step.

By a "modulatory compound", as used herein, is meant any compound capable of either decreasing $\alpha 6\beta 1$ expression (i.e., at the level of transcription,

translation, or post-translation) or decreasing α6β1 protein activity (i.e., the amount of activity per unit of α6β1 protein).

By a "differentiation agent" is meant any compound which, when added to cells in vitro or introduced into a mammal, result in a change in the phenotype of a cell or tissue, including the expression of one or more markers indicative of a particular stage in the cell's or tissue's life cycle. Differentiation agents include, without limitation, retinoic acid and cyclic AMP.

## Detailed Description of the Drawings

Fig. 1 is a graph of surface expression of integrin subunits on MDA MB-435 cells. MDA MB-435 cells were analyzed by flow cytometry using MAbs specific for the indicated integrin subunits. The left scan in each profile was obtained using a nonspecific mouse IgG, and the right scan corresponds to the expression levels of the specified integrin subunits.

Figs. 2A and 2B are graphs of mAb inhibition of MDA MD-435 adhesion and migration on laminin and collagen I. 2A shows adhesion to tissue culture wells were coated with laminin (20 μg/ml) or collagen 1 (20 μg/ml). MDS MB-435 cells were preincubated with antibodies to specific integrin subunits before addition to the protein-coated wells. After a 30 min. incubation at 37°C, nonadherent cells were removed by washing, and adherent cells were fixed, stained, and quantitated as described below. The data shown are the mean values +SEM (bars) of three experiments done in duplicate. Fig. 2B shows migration data for MDA MB-435 cells preincubated with antibodies to specific integrin subunits before addition to Transwell chambers. Media containing either laminin (15 μg/ml) or collagen I (μg/ml) were added to the bottom wells. After a 4 hr. incubation at 37°C, the

cells that had not migrated were removed, and the cells that had migrated onto the lower surface of the filter were fixed, stained, and quantitated. The data shown are the mean values +SEM (bars) from two experiments done in duplicate, laminin; collagen I.

Figs. 3A-3C depict expression of the truncated B4-ΔCYT integrin subunits in MDA MB-435 cells. Fig. 3A is a schematic demonstration of the strategy used to knock out α6β1. This strategy is based on the finding that α6 associates preferentially with β4 in comparison with β1. Thus, expression of the β4-ΔCYT subunit will result in formation of the α6β4-ΔCYT heterodimer at the expense of α6β1 association. Because of its cytoplasmic domain deletion, the α6β4-ΔCYT integrin should be a null receptor unable to mediate laminin interactions. Presumably, the excess β1 that arises from this process can associate with α subunits. Fig. 3B show profiles obtained when a population of transfected MDA MB-435 cells expressing β4-ΔCYT on the cell surface were isolated by FACS using UM-A9, a mAb specific for the β1 integrin subunit. The resulting cells were analysed by flow cytometry. The left scan in each profile was obtained using a nonspecific mouse IgG, and the right scan corresponds to the expression levels of the specified integrin subunits, Mock, MDA MB-435 cells transfected with the vector alone: β4-ΔCYT, MDA MB-435 cells transfected with the β4-ΔCYT cDNA. Fig. 3C shows immunoprecipitation of transfected cells. The cells were surface labeled with biotin, and aliquots of detergent extracts from equal numbers of cells were immunoprecipitated with either the 2B7 or UM-A9 mAbs. Immunoprecipicies were resolved by 8% SDS-PAGE under reducing conditions and transferred to nitrocellulose filters. Proteins were visualized with streptavidin conjugated to horseradish peroxidase and enhanced

chemiluminescence. Lane 1, mock transfectants immunoprecipitated with 2B7; Lane 2, β4-ΔCYT transfectants immunoprecipitated with UM-A9; Lane 3, mock transfectants immunoprecipitated sequentially with UM-A9 and then with 2B7; Lane 4, β4-ΔCYT transfectants immunoprecipitated sequentially with UM-A9 and then 2B7. The migration positions of the β1, α6 and β4-ΔCYT subunits are shown in the left margin. The molecular mass markers are indicated in the right margin.

Figs. 4A-C show functional assays using the β4-ΔCYT MDA MB-435 transfectants. Fig. 4A is a graph of adhesion of MDA MB-435 transfectants. Cells were assayed for their ability to adhere to laminine and collagen I subunits. Transfected cells (109) were added to laminin-coated (20 μg/ml) and collagen I-coated (20 μg/ml) wells and incubated for 30 min. at 37°C. Nonadherent cells were removed by washing, and subsequent cells were fixed, stained and quantitated. The data shown are the mean values +SEM (bars) of these experiments done in triplicate. Fig. 4B is a graph of migration of MDA MB-435 transfectants. Cells were assayed for their ability to migrate toward laminin and collagen I. Media containing either laminin (15 μg/ml) or collagen I (15 μg/ml) were added to the bottom wells of Traswell chambers and the transfectants ($10^5$) were added to the top wells. After a 4 hr. incubation at 37°C, the cells that had not migrated were removed, and the cells that had migrated onto the lower surface of the filter were fixed, stained and quantitated. The data shown are the mean values +SEM (bars) from two experiments done in duplicate. Fig. 4C is a graph of invasion by MDA MB-435 transfectants. Cells were assayed for their ability to invade Marrigel. Marrigel was diluted in cold distilled water, added to the upper well of Transwell chambers and dried under a sterile hood. The Marrigel was

reconstructed with medium, and the transfectants (2 x 109) were added to each well. Conditioned NIH-3T3 medium was added to the bottom wells of the chambers. After 5-7 hr. at 37°C, the cells that had not invaded were removed, and the cells that had invaded to the lower surface of the filters were fixed, stained and quantitated as described in "Materials and Methods".

## Detailed Description

### Introduction

The involvement of the α6β1 integrin in breast carcinoma progression has been the focus of many recent studies. However, a survey of literature in this area reveals that the potential involvement of this laminin receptor in breast carcinoma remains the subject of considerable debate. Several immunohistochemical studies have concluded that the expression of α6β1 is diminished in primary breast carcinomas. Such studies, although potentially interesting, are limited in their significance by a number of factors. For example, a common observation is that in normal mammary glands, the cells with the strongest α6 immunoreactivity are myoepithelial cells and not the ductal epithelial and acinar cells that give rise to the majority of breast cancers. Thus, the conclusion that the α6 integrin is diminished in breast cancers may be misleading, because it is based on comparing the staining intensity of diverse populations of cells. A second factor is that the pathological specimens used in such studies are often chosen at random, with no reference to either disease stage or other clinical parameters.

The above observations emphasize the need for functional studies on the role of the α6β1 integrin in breast carcinoma progression. To address this issue, we have assessed the expression and function of this

integrin in human breast carcinoma. Here we provide data showing that a human breast carcinoma cell line, MDA-MB-435, known to be highly invasive and metastatic (Price et al., *Cancer Res.*, 50:717-721, 1990), expresses three potent integrin laminin receptors: $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha 6\beta 1$ (Mercurio, *Trends Cell Biol.* 5:419-423, 1993). Antibody inhibition studies, however, revealed that these cells use only $\alpha 6\beta 1$ to mediate adhesion and migration on laminin matrices. To investigate further whether $\alpha 6\beta 1$ may be involved in the aggressive behavior of MDA-MB-435 cells, we developed a dominant-negative strategy for knocking out $\alpha 6\beta 1$ function in these cells. This strategy involves expression of a cytoplasmic domain deletion mutant of the $\beta 4$ integrin subunit by cDNA transfection. Stable transfectants of MDA-MB-435 cells that expressed this mutant $\beta 4$ subunit were inhibited dramatically in their ability to adhere and migrate on laminin matrices. In addition, the capacity of these cells to invade through a basement membrane matrix was also reduced significantly. Because *in vitro* invasive properties correlate strongly with *in vivo* behavior of carcinoma cell lines (Albini et al., *Cancer Res.* 47:3239-3245, 1987), these findings provide functional data to support the idea that the $\alpha 6\beta 1$ integrin is important for breast cancer progression. Moreover, this dominant-negative approach is a powerful method that should be useful in assessing the role of the $\alpha 6\beta 1$ integrin in other cell types.

In addition to our *in vitro* results, we have used the MDA-MB-435 cell line expressing the cytoplasmic domain deletion mutant of the $\beta 4$ integrin subunit to demonstrate loss of metastic potential *in vivo*.

Our results indicate that $\alpha 6\beta 4$ integrin can be used as a diagnostic and prognostic antibody. In addition, therapies which eliminate $\alpha 6\beta 1$ from the cell

surface or block α6β1 function may be used to slow, halt, or prevent metastisis of breast cancer. The following examples are provided to illustrate the invention. They are not intended to limit the scope of the invention.

Anti-α6β1 Antibodies

To generate α6β1-specific antibodies, a α6β1 coding sequence (i.e., the whole protein or the ctyoplasmic region may be expressed as a C-terminal fusion with glutathione S-transferase (GST) (Smith et al., Gene 67:31-40, 1988). The fusion protein may be purified on glutathione-Sepharose beads, eluted with glutathione cleaved with thrombin (at the engineered cleavage site), and purified to the degree necessary for immunization of rabbits. Primary immunizations may be carried out with Freund's complete adjuvant and subsequent immunizations with Freund's incomplete adjuvant. Antibody titres are monitored by Western blot and immunoprecipitation analyses using the thrombin-cleaved α6β1 protein fragment of the GST-α6β1 fusion protein. Immune sera may be affinity purified using CNBr-Sepharose-coupled α6β1 protein. Antiserum specificity is determined using a panel of unrelated GST proteins (e.g., GSTp53, Rb, HPV-16 E6, and E6-AP) and GST-trypsin (which may be generated by PCR using known sequences).

As an alternate or adjunct immunogen to GST fusion proteins, peptides corresponding to relatively unique hydrophilic regions of α6β1 may be generated and coupled to keyhole limpet hemocyanin (KLH) through an introduced C-terminal lysine. Antiserum to each of these peptides is similarly affinity purified on peptides conjugated to BSA, and specificity tested in ELISA and Western blots using peptide conjugates, and by Western blot and immunoprecipitation using α6β1 expressed as a GST fusion protein.

Alternatively, monoclonal antibodies may be prepared using the α6β1 proteins described above and standard hybridoma technology (see, e.g., Kohler et al., Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, 1981; Ausubel et al., supra). Once produced, monoclonal antibodies are also tested for specific α6β1 recognition by Western blot or immunoprecipitation analysis (by the methods described in Ausubel et al., supra). Antibodies which specifically recognize α6β1 are considered to be useful in the invention; such antibodies may be used, e.g., in an immunoassay to monitor the level of α6β1 produced by a mammal (for example, to determine the amount or location of α6β1).

Preferably, antibodies of the invention are produced using fragments of the α6β1 protein which lie outside highly conserved regions and appear likely to be antigenic, by criteria such as high frequency of charged residues. In one specific example, such fragments are generated by standard techniques of PCR and cloned into the pGEX expression vector (Ausubel et al., supra). Fusion proteins are expressed in E. coli and purified using a glutathione agarose affinity matrix as described in Ausubel et al. (supra). To attempt to minimize the potential problems of low affinity or specificity of antisera, two or three such fusions are generated for each protein, and each fusion is injected into at least two rabbits. Antisera are raised by injections in a series, preferably including at least three booster injections.

Identification and Administration of Molecules that Modulate α6β1 Protein Expression

Use of the α6β1 cDNA facilitates the identification of molecules which increase or decrease α6β1 expression. According to one approach, candidate molecules are added at varying concentrations to the culture medium of cells expressing α6β1. α6β1 expression is then measured, for example, by standard Northern blot analysis (Ausubel et al., supra) using a α6β1 cDNA (or cDNA fragment) as a hybridization probe. The level of α6β1 expression in the presence of the candidate molecule is compared to the level measured for the same cells in the same culture medium but in the absence of the candidate molecule.

If desired, the effect of candidate modulators on expression may, in the alternative, be measured at the level of α6β1 protein production using the same general approach and standard immunological detection techniques, such as Western blotting or immunoprecipitation with a α6β1-specific antibody (for example, the α6β1 antibody described herein).

Candidate modulators may be purified (or substantially purified) molecules or may be one component of a mixture of compounds (e.g., an extract or supernatant obtained from cells; Ausubel et al., supra). In a mixed compound assay, α6β1 expression is tested against progressively smaller subsets of the candidate compound pool (e.g., produced by standard purification techniques, e.g., HPLC or FPLC) until a single compound or minimal compound mixture is demonstrated to modulate α6β1 expression.

Candidate α6β1 modulators include peptide as well as non-peptide molecules (e.g., peptide or non-peptide molecules found, e.g., in a cell extract, mammalian

serum, or growth medium on which mammalian cells have been cultured).

A molecule which promotes a decrease in $\alpha 6\beta 1$ expression or $\alpha 6\beta 1$ activity is considered particularly useful in the invention; such a molecule may be used, for example, as a therapeutic to dencrease cellular levels of $\alpha 6\beta 1$.

Modulators found to be effective at the level of $\alpha 6\beta 1$ expression or activity may be confirmed as useful in animal models and, if successful, may be used as anti-cancer therapeutics.

A $\alpha 6\beta 1$ modulator may be administered with a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer $\alpha 6\beta 1$ to patients suffering from or presymptomatic for a $\alpha 6\beta 1$-associated carcinoma. Any appropriate route of administration may be employed, for example, parenteral, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable

lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for α6β1 modulatory compounds include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

If desired, treatment with a α6β1 modulatory compound may be combined with more traditional cancer therapies such as surgery, radiation, or chemotherapy.

Detection of A Malignant Condition

α6β1 polypeptides and nucleic acid sequences find diagnostic use in the detection or monitoring of breast carcinoma. In particular, because α6β1 is involved in the control of cell division and because the absence of α6β1 correlates with the development of carcinomas in humans, a increase in the level of α6β1 production provides an indication of a malignant or pre-malignant condition. Levels of α6β1 expression may be assayed by any standard technique. For example, its expression in a biological sample (e.g., a biopsy) may be monitored by standard Western blot analysis or may be aided by PCR (see, e.g., Ausubel et al., supra; PCR Technology: Principles and Applications for DNA Amplification, ed., H.A. Ehrlich, Stockton Press, NY; and Yap and McGee, Nucl. Acids. Res. 19:4294, 1991).

In yet another approach, immunoassays are used to detect or monitor α6β1 protein in a biological sample. α6β1-specific polyclonal or monoclonal antibodies (produced as described above) may be used in any standard

immunoassay format (e.g., ELISA, Western blot, or RIA assay) to measure α6β1 polypeptide levels; again comparison is to wild-type α6β1 levels, and a decrease in α6β1 production is indicative of a malignant condition. Examples of immunoassays are described, e.g., in Ausubel et al., supra. Immunohistochemical techniques may also be utilized for α6β1 detection. For example, a tissue sample may be obtained from a patient, and a section stained for the presence of α6β1 using an anti-α6β1 antibody and any standard detection system (e.g., one which includes a secondary antibody conjugated to horseradish peroxidase). General guidance regarding such techniques can be found in, e.g., Bancroft and Stevens (Theory and Practice of Histological Techniques, Churchill Livingstone, 1982) and Ausubel et al. (supra).

α6β1 Therapy

The methods of the instant invention may be used to reduce or diagnose the disorders described herein in any mammal, for example, humans, domestic pets, or livestock. Where a non-human mammal is treated or diagnosed, the α6β1 polypeptide, nucleic acid, or antibody employed is preferably specific for that species.

Examples

**I. Materials and Methods**

*Cells and Antibodies.* The MDA-MB-435 breast carcinoma cell line was obtained from the Lombardi Breast Cancer Depository (Georgetown University). The cells were grown in DMEM (GIBCO) supplemented with 10% FCS (GIBCO) and 1% penicillin-streptomycin (GIBCO). The antibodies specific for integrin subunits were as follows: mAb[3] 13 (β1); UM-A9 (β4); IIE10 (α2); IVA5 (α3); and 2B7 (α6) (Shaw et al., *J. Biol. Chem.*

268:11401-11408, 1993); and Mouse IgG was obtained from Sigma Chemical Co.

*Transfections*. The construction of the β4 cytoplasmic deletion mutant cDNA and its insertion into the pcDNA3 eukaryotic expression vector have been described previously (Clarke et al., *J. Biol. Chem.* 270:22673-22676, 1995). The pcDNA3 vector containing the mutant β4 cDNA, as well as the vector alone, were transfected into the MDA-MB-435 cell line using lipofectin (GIBCO) according to the instructions of the manufacturer. Neomycin-resistant cells were isolated by selective growth in medium containing G418 (0.6 mg/ml; GIBCO). The stable transfectants were pooled, and a population of cells that expressed the human β4 subunit on the cell surface was isolated by FACS. A human β4 integrin-specific mAB, UM-A9, was listed for this sorting and for subsequent analysis of the transfectants (Clarke et al., *J. Biol. Chem.* 270:22673-22676, 1995). The sorting was repeated sequentially to enrich for homogeneous populations of cells expressing high levels of the transfected β4-ΔCYT subunit on the cell surface.

*Flow Cymmetry*. Transfected MDA-MB-435 cells were washed twice with RPMI 1640 media containing 25 mm HEPES (RPMI-H) and 0.2% BSA (RH/BSA). Aliqouts of cells (5 x $10^5$) were incubated for 45-60 min at room temperature with RH/BSA containing UM-A9 (3 μg/ml). The cells were washed two times with RH/BSA and then incubated with goat $F(ab')_2$ antimouse IgG coupled to FITC (Iago) for 45-60 min at room temperature. After washing two times with RH/BSA, the cells were resuspended in the same buffer and analyzed using a FACScm (Becton Dickinson).

*Functional Assays*. Adhesion assays were performed as described previously (Shaw et al., *J. Biol. Chem.* 268:11401-11408, 1993). Briefly, multiwell tissue culture plates (11.3-mm diameter) were coated overnight

at 4°C with 0.2 ml PBS (20 μg/ml) containing either murine Engelbreth-Holm-Swain laminin or rat collagen I. The wells were then washed with PBS, and $10^5$ cells in RPMI-H containing 1% DSA were added. After a 30-min incubation at 37°C, the wells were washed three times with RPMI-H at 37°C, fixed for 15 min with methanol and stained with a 0.2% solution of crystal violet in 2% ethanol. The crystal violet strain was solubilized with a 1% solution of SDS, and adhesion was quantitated by measuring the A at 600 nm. To examine inhibition of adhesion, cells were preincubated for 30 min at room temperature in the presence of antibodies before addition to the assay wells.

Cell migration assays were performed using 6.5-mm Transwell chambers (8-μm pore size; Costar) as described previously (Shaw and Mercurio, *Mol. Biol. Cell* 5:679-690, 1994). Briefly, RPMI-H containing 15 μg/ml laminin (0.6 ml) was added to the bottom well, and the filters were coated for -30 min at 37°C. Cells were resuspended in the appropriate buffer at a concentration of $10^5$/ml, and $10^5$ cells were added to the top well of the Transwell chambers. After a 4-h incubation, the cells that had not migrated were removed from the upper face of the filters using cotton swabs, and the cells that had migrated to the lower surface of the filters were fixed in methanol and stained with a 0.2% solution of crystal violet in 2% ethanol. Migration was quantitated by counting using bright-field optics with a Nikon Diaphot microscope equipped with a 16-square reticle. The surface area of this grid was determined to be 1 $mm^2$. Five separate fields were counted for each filter. To examiner the inhibition of migration, cells were preincubated for 30 min at room temperature in the presence of antibodies before addition to the assay wells.

Matrigel invasion assays were performed as described (Albini et al., *Cancer Res.* 47:3239-3245, 1987) using 6.5-mm Transwell chambers (8-μm pore size). Matrigel, purified from the Engelbreth-Holm-Swam tumor (Kleinman et al., *Biochemistry* 25:312-318, 1986), was diluted in cold distilled water, added to the Transwells (6.25-12.5 μg/well), and dried under a sterile hood. The Marigel was then reconstituted with medium for 2 h at 37°C before the addition of cells. Cells were resuspended in serum-free medium containing 0.1% BSA, and 2 x $10^5$ cells were added to each well. Conditioned NIH-373 medium was added to the bottom wells of the chambers. After 5-7 h, the cells that had not invaded were removed from the upper face of the filters using cotton swabs, and the cells that had invaded to the lower surface of the filters were fixed in methanol and stained with a 0.2% solution of crystal violet in 2% ethanol. Invasion was quantitated as described above for migration assays.

*Surface Labeling and Immunoprecipitation.* Cells were trypsinized from their dishes and washed twice with PBS. After washing, the cells were resuspended in the same buffer at a concentration of 5 x $10^5$ cells/ml, NHS-LC-biotin (Pierce Chemical Co., Rockville, IL) was resuspended in DMSO and added to the cells at a concentration of 0.1 mg/ml. Cells were incubated in the presence of biotin for 1 hour at room temperature with gentle shaking. Subsequently, the cells were washed several times with PBS containing 50 mM $NH_4Cl$ to remove unincorporated biotin. Surface biotinylated cells were solubilized at 4°C for 15 min in 10 mM Tris buffer (pH 8) containing 0.15 M NaCl, 1% Triton-X-100, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin and pepstatin, and 50 μg/ml leupeptin. Nuclei were removed by centrifugation at 12.000 x g for 10 min. Aliquots of labeled cell extracts from an equivalent number of cells

were incubated for 1 h at 4°C with α6-or β4-specific antibodies. Immune complexes were recovered with protein G agarose (Pharmacia). The agarose beads were added for 1 h at 4°C with constant agitation. For immunodepletion experiments, the cell extracts were immunoprecipitated initially with β4-specific antibodies, and the supernatant was immunoprecipitated subsequently with α6-specific antibodies. The beads were washed two times with 50 mM Tris buffer (pH 7.5) containing 0.15M NaCl and 0.1% Triton X-100. two times with the same buffer containing 0.5 M NaCl, and one time with 0.05 M Tris (pH 6.8). Laemmeli sample buffer was added to the samples, which were then incubated at 100°C for 4 min. Surface biotinylated immunoprecipitates were resolved by SDS-Page (8%) and transferred to ainocellulose filters. The filters were blocked for 30 min. using 50 mM Tris buffer (pH 7.5) containing 0.5 M MaCl, 0.1% Tween 20, and 5% (w/v) Carnation dry milk. The filters were incubated for 1 h in the same buffer containing streptavidin conjugated to horseradish peroxidase (2 μg/ml; Pierce). After three 10-min washes in blocking buffer lacking dry milt, protein was detected by enhanced chemiluminescence (Amersham).

**II. Integrin Laminin Receptor Expression and Usage in MDA-MB 435 Cells.**

MDA-MB-435 cells were analyzed by flow cytometry to determine the cell surface expression of integrin laminin receptor subunits. As shown in Fig. 1, these cells express the α2, α3, α6, and β1 subunits, but they lack expression of the β4 subunit. These data indicate that three possible integrin laminin receptors are expressed on the cell surface by this cell line: α2β1, α3β1, and α6β1. However, expression of an integrin receptor on the cell surface does not always correlate with functional activity (Mercurio, Trends. Cell Biol.

5:419-423, 1995). To assess whether these receptors participated in mediating laminin interactions, adhesion assays were performed in the presence of inhibitory antibodies specific for each of the integrin subunits. Antibodies that recognize the α6 and β1 subunits inhibited adhesion to laminin almost completely (Fig. 2A). In contrast, function-blocking antibodies specific for the α2 and α3 subunits had no inhibitory effect on laminin adhesion compared with the control mouse IgG antibody. The α2-specific MAb did inhibit adhesion to collagen I, however (Fig. 2A).

One property considered important for metastatic cells is their ability to migrate on laminin matrices (?? et al., Amer. Rev. Biochem. 55:1037-1057, 1986). As shown in Fig. 2B, MDA-MB-435 cells exhibited significant migration toward a laminin gradient in a 4-h modified Boyden chamber assay. In fact, this rate of migration is considerably faster than the rates of many other carcinoma cell lines we have examined. Function-blocking experiments revealed that only α6- and β1-specific antibodies inhibited this migration on laminin (Fig. 2B). Taken together, these data demonstrate that the α6β1 integrin is essential for mediating both the adhesion and migration of MDA-MB-435 cells on laminin.

**III. Use of a Dominant-Negative Transfection Strategy to Knock Our α6β1 Function in MDA-MB-435 Cells.**

To assess the contribution for the laminin receptor function of α6β1 to the behavior of MDA-MB-435 cells more rigorously, we designed a dominant-negative approach to eliminate, or knock out, the expression of this receptor. Our strategy was based on the findings that the β4 subunit associates exclusively with the α6 subunit, and α6 associates preferentially with β4 in comparison with β1 (Hemler et al., *J. Biol. Chem.* **264**: 6529-6535, 1989). Thus, we hypothesized that if a β4

subunit lacking its cytoplasmic domain ($\beta$4-$\Delta$CYT) was expressed in these cells, it would associate with the $\alpha$6 subunit on the cell surface and create a nonfunctional receptor ($\alpha$6$\beta$4-$\Delta$CYT). This expression also should decrease the amount of $\alpha$6$\beta$1 on the cell surface, eliminating the only functional laminin receptor in these cells (Fig. 3A).

MDA-MB-435 cells were transfected with the $\beta$4-$\Delta$CYT cDNA and a population of stably transfected cells that expressed high surface levels of $\beta$4 were obtained by FACS (Fig. 3B). The $\beta$4-$\Delta$CYT subunit associated with the $\alpha$6 subunit on the cell surface as determined by surface biotinylation and immunoprecipitation with antibodies specific for $\alpha$6 and $\beta$4 (Fig. 3C). The total level of $\alpha$6 on the cell surface was not altered in the $\beta$4-$\Delta$CYT transfectants (Fig. 3B). However, the amount of $\alpha$6$\beta$1 receptor present on the cell surface was decreased by the expression of the $\beta$4-$\Delta$CYT cDNA, as shown in Fig. 3C, after removing $\beta$4 from cell lysates by immunodepletion, very little $\alpha$6 remained in the $\beta$4-$\Delta$CYT lysates in comparison to the amount obtained from the mock transfectant lysates. The small amount of $\alpha$6 that is seen in the $\beta$4-$\Delta$CYT transfectant lysates may be residual $\alpha$6$\beta$4-$\Delta$CYT, because some $\beta$4-$\Delta$CYT is present in this $\alpha$6 immunoprecipitation. By FACS analysis, the level of $\alpha$2$\beta$1 on the surface of the $\beta$4-$\Delta$CYT transfectants was comparable to the mock transfectants, whereas an increase in $\alpha$3$\beta$1 surface expression was observed (data not shown).

MDA-MB-435 cells that had been transfected with either the vector alone or the $\beta$4-$\dot{\eta}$CYT cDNA were analyzed for their ability to adhere and migrate on laminin. As shown in Figs. 4A and 4B, cells that expressed the truncated $\beta$4 subunit were inhibited almost completely in their ability to both adhere and migrate on laminin. In contrast, their adhesion and migration on collagen I did

not differ significantly from the mock transfectants, indicating that the inhibition wa specific for laminin. In control experiments, the mock and β4-ΔCYT transfectants also adhered comparably to uncoated Transwell filters. It can be concluded from these data that the α6β4-ΔCYT receptor cannot mediate normal adhesive interactions with a laminin substratum, and that the β4-ΔCYT subunit is functioning in a dominant-negative manner for α6β1.

To begin to assess how the loss of laminin-binding function might influence the invasive properties of this metastatic cell line, *in vitro* invasion assays were performed. The mock and β4-ΔCYT transfectants were assayed for their ability to invade through Transwell filters that had been coated on the upper surface with Matrigel. As shown in Fig. 4C, the mock transfectants invaded through the matrix at levels that have been demonstrated previously for the parental MDA-MB-435 cell line (Brunner et al., *Eur. J. Cancer*, 28A:1989-1995, 1992). However, expression of the β4-ΔCYT subunit in these cells reduced Matrigel invasion by ~68% (Fig. 4C). We conclude from these data that α6β1 is critical for the invasive behavior of these cells.

## IV. Loss of MDA-MB-435 Metastic Potential In Vivo

TABLE I

MDA-435 BREAST CARCINOMA CELLS INITIAL NUDE MICE DATA

| Subclone | Ascites | Degree of Peritoneal Spread | | |
|---|---|---|---|---|
| (extensive/moderate/negative) | | | | |
| Mock-B1 | 5/5 | 5/5 | 0/5 | 0/5 |
| Mock-B6 | 4/7 | 4/7 | 2/7 | 1/7 |
| **TOTAL MOCK** | **9/12** | **9/12** | **2/12** | **1/12** |
| β4-ΔCYT-1A2 | 0/3 | 0/3 | 1/3 | 2/3 |
| β4-ΔCYT-3C12 | 1/7 | 0/7 | 2/7 | 5/7 |
| **TOTAL β4-ΔCYT** | **1/10** | **0/10** | **3/10** | **7/10** |

-Extensive Metastatic Foci Evident in Lungs and Liver of Mock Transfectants
-Few, if any, Foci Evident in β4-ΔCYT Transfectants

## V. Discussion of Experimental Results

Our results provide evidence to support the involvement of the integrin α6β1 in the aggressive behavior of breast carcinoma cells. Specifically, we observed that MDA-MB-435 cells, a highly invasive and metastatic breast carcinoma cell line, use only α6β1 to interact with laminin matrices, although they express other potential integrin laminin receptors. More convincingly, we developed a strategy to knock out α6β1 function in these cells by expression of a dominant-negative receptor (α6β4-ΔCYT). MDA-MB-435 cells that expressed this dominant-negative receptor were inhibited drastically in their ability to adhere and migrate on laminin matrices. In addition, such cells invaded

Matrigel substantially less well than either wild-type cells or mock transfectants. This *in vitro* invasion assay has been shown to provide a strong indication of invasive potential *in vivo* (Albini et al., *Cancer Res.*, 47: 3239-3245, 1987).

In its capacity as a laminin receptor, a role for the $\alpha 6\beta 1$ integrin in the invasive processes of carcinoma cells appears quite feasible. It has been known for some time that laminin plays a key role in carcinoma invasion and metastasis (Kleinman et al., Biochemistry 25:312-318, 1986; Albrechtsen et al., Pathol. Annu. 21:251-276, 1986; Kleinman et al., The Laminins: 47:161-86, 1993). Earlier studies emphasized the need for carcinoma cells to penetrate epithelial basement membranes as a prelude to stromal invasion, and more recent studies have highlighted the possibility that invasive carcinoma cells may deposit their own laminin matrix to promote their egress (Miyazaki, et al., *Proc. Natl. Acad. Sci. USA*, 90: 11767-11771, 1993). Within this context, our data on the expression and function of $\alpha 6\beta 1$ in metastatic breast carcinoma cells support a key role for this integrin in these laminin mediated invasive processes.

Most likely, the marked diminution in laminin adhesion, migration, and invasion observed in the $\beta 4$-$\Delta$CYT transfectants can be attributed to the elimination of $\alpha 6\beta 1$ expression on the cell surface. This assumption is based on our finding that the parental MDA-435 cells use only $\alpha 6\beta 1$ to interact with laminin, although they express other potential laminin receptors, including $\alpha 2\beta 1$ and $\alpha 3\beta 1$. For this reason, it does not seem likely that the overexpression of $\alpha 3\beta 1$ that we observed in the $\beta 4$-$\Delta$CYT transfectants is directly responsible for the inhibition of laminin-mediated interactions. The possibility exists, however that in addition to the elimination of $\alpha 6\beta 1$ expression, the expression of the $\beta 4$-$\Delta$CYT cDNA could

influence other unknown factors (e.g., interacting molecules) that might contribute to the functional differences observed in the transfectants. In any case, it is apparent that neither α6β4-ΔCYT nor α3β1 has the ability to mediate interactions with laminin based on our functional data.

Aside from its relevance to breast carcinoma, the dominant-negative transfection strategy described in this study should be a powerful tool for knocking out the function of α6β1 specifically in other cell types that do not express α6β4. Such an approach could be extremely useful, because α6β1 has been implicated in a myriad of biological and pathological phenomena (Mercurio et al., *Trends, Cell Biol.*, 5:419-423, 1995). Moreover, expression of this dominant-negative receptor can be used to dissect the functions of α6β1 in cells that express multiple integrin laminin receptors. For example, RKO rectal carcinoma cells use both the α6β1 and α2β1 receptors for mediating laminin interactions. Expression of the truncated β4 subunit in the RKO cell line does not inhibit their adhesion to laminin completely because of the presence of α2β1. However, it does decrease their ability to migrate on laminin markedly, an observation that supports the notion that these cells use primarily α6β1 for migration.

In addition to use of antibodies to full-length polypeptides, the invention also includes use of antibodies to α6β1 polypeptide fragments. As used herein, the term "fragment," means at least 20 contiguous amino acids, preferably at least 30 contiguous amino acids, more preferably at least 50 contiguous amino acids, and most preferably at least 60 to 80 or more contiguous amino acids. Fragments of α6β1 polypeptides can be generated by methods known to those skilled in the art or may result from normal protein processing (e.g.,

removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events).

Preferable fragments or analogs according to the invention are those which facilitate specific detection of a $\alpha 6\beta 1$ polypeptide or polypeptide levels in a sample to be diagnosed.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

Other embodiments are within the following claims.

What is claimed is:

1. A method of detecting an increased likelihood of metastic breast cancer in a patient, said method comprising comparing the levels of $\alpha 6\beta 1$ integrin present in the breast cells of said patient to the levels of $\alpha 6\beta 1$ integrin in the breast cells from an unaffected individual, and increase in said $\alpha 6\beta 1$ integrin levels in said cell of said patient relative to said levels in cells from said unaffected individual indicating an increased likelihood of metastic breast cancer.

2. The method of claim 1, wherein said increase in $\alpha 6\beta 1$ integrin levels is two-fold or greater.

3. A method of prophylaxis or treatment of breast cancer in a patient, said method comprising inhibition of of $\alpha 6\beta 1$ integrin function.

4. The method of claim 3, wherein said inhibition is by administering an antibody which binds the extracellular domain of $\alpha 6\beta 1$ integrin.

5. The method of claim 3, wherein said inhibition is by administering a dominant negative nucleic acid construct.

6. The method of claim 5, wherein said construct encodes an $\alpha 6\beta 1$ polypeptide comprising the transmembrane and extracellular domains of $\alpha 6\beta 1$, said polypeptide having a deletion of at least a portion of the cytoplasmic domain.

7. The method of claim 3, wherein said inhibition is by administering a polypeptide comprising the $\alpha 6\beta 1$ transmembrane and cytoplasmic domains.

8. The method of claim 3, wherein said inhibiting is by the administering of laminin or a fragment thereof.

9. The method of claim 8, wherein said fragment is from the G domain of laminin.

10. A kit for diagnosing a mammal for the presence of breast carcinoma or an increased likelihood of developing a breast carcinoma, said kit comprising a substantially pure antibody that specifically recognizes and binds a $\alpha 6\beta 1$ polypeptide.

11. The kit of claim 10, further comprising means for detecting said binding of said antibody to said $\alpha 6\beta 1$ polypeptide.

12. A kit for diagnosing a mammal for the presence of a malignancy or an increased likelihood of developing a malignancy, said kit comprising a substantially pure wild-type $\alpha 6\beta 1$ polypeptide.

13. A method of identifying a modulatory compound which is capable of decreasing $\alpha 6\beta 1$ levels, comprising (a) providing a cell expressing said $\alpha 6\beta 1$ polypeptide; and (b) contacting said cell with a candidate compound, a decrease in said $\alpha 6\beta 1$ protein levels following contact with said candidate compound identifying a modulatory compound.

14. A method of treating a mammal with a disease involving increased expression of a $\alpha 6\beta 1$, said method comprising administering to said patient a modulatory compound that is capable of decreasing $\alpha 6\beta 1$ protein

levels, in an amount sufficient to reduce the symptoms of said disease in said mammal.

15. The use of an antibody which specifically binds $\alpha 6\beta 1$ integrin for the manufacture of a kit for the diagnosis of malignant breast carcinoma.

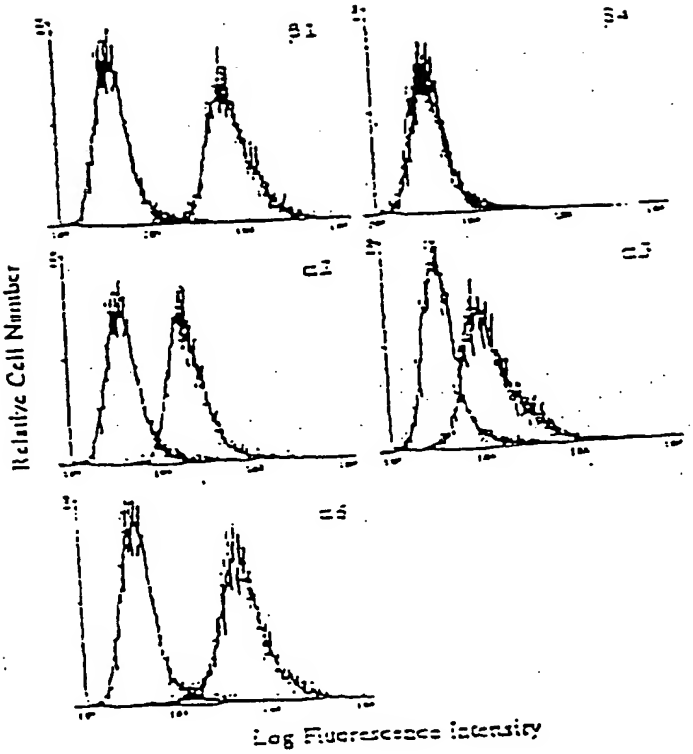


Fig. 1

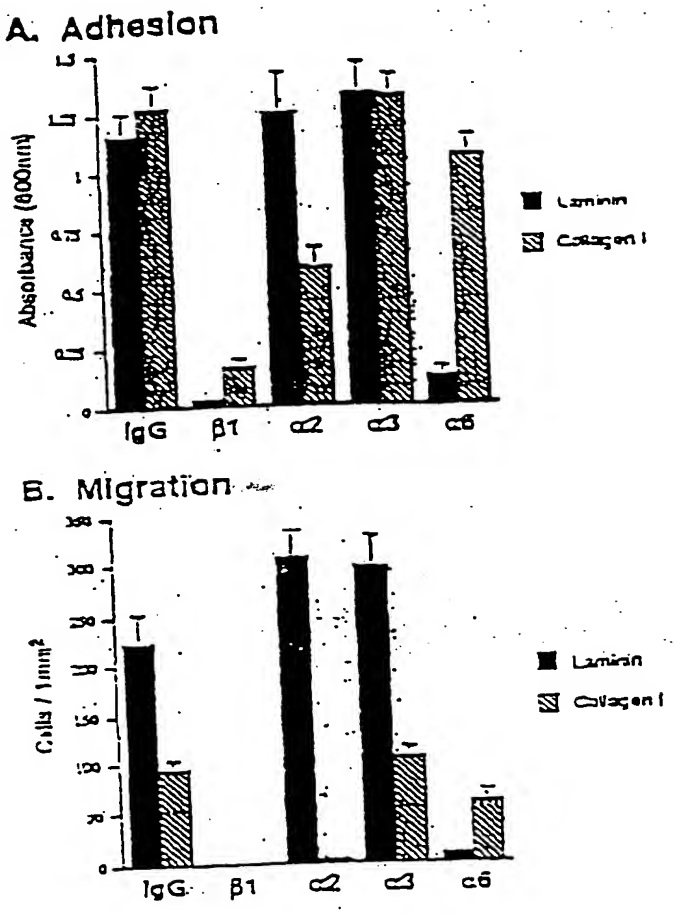
A. Adhesion
Absorbance (600nm)
IgG
β1
α2
α3
α6
Laminin
Collagen I
B. Migration
Cells / 1mm²
IgG
β1
α2
α3
α6
Laminin
Collagen I

Fig. 2

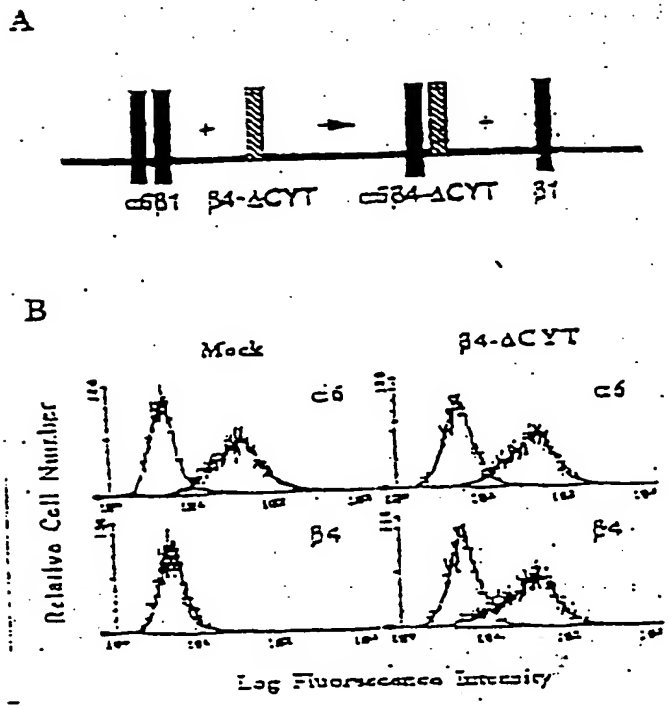
A
α6β1
β4-ΔCYT
α6β4-ΔCYT
β1
B
Mock
β4-ΔCYT
α6
α6
β4
β4
Relative Cell Number
Log Fluorescence Intensity
C
1 2 3 4
200
β1
α6
116
β4ΔCyt
97

Fig. 3

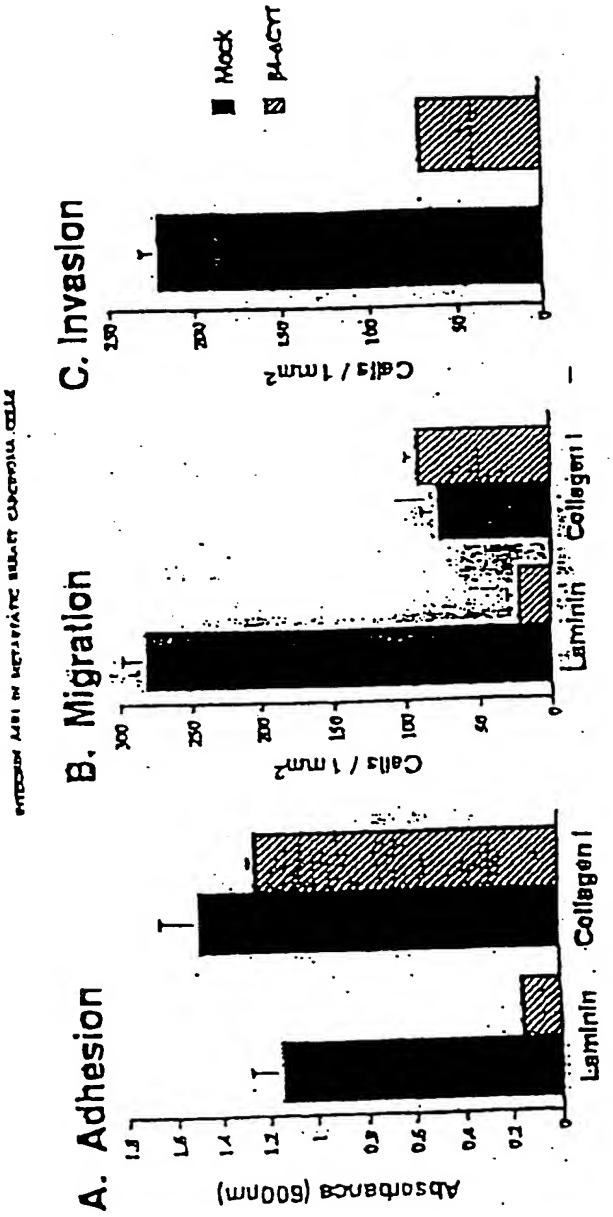
A. Adhesion
Absorbance (500nm)
Laminin
Collagen I
B. Migration
Cells / 1 mm²
Laminin
Collagen I
C. Invasion
Cells / 1 mm²
Mock
pLACZ

Fig. 4

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/13691

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 39/395; G01N 33/53
US CL :424/130.1, 143.1, 155.1, 174.1; 435, 7.1, 7.23

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 143.1, 155.1, 174.1; 435, 7.1, 7.23

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, BIOSIS, EMBASE, MEDLINE, CA, WPI
search terms: alpha6beta1, breast, carcinoma, cancer, tumor

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Eur. J. Cancer, Volumn 29, Suppl. 6, issued 1993, Imhof et al., "Integrins and Metastasis", page S22, see Abstract No. 85. | 1-15 |
| Y | Cancer Research, Volume 55, issued 15 February 1995, Friedrichs et al., "High Expression Level of alpha6 Integrin in Human Breast Carcinoma Is Correlated with Reduced Survival", pages 901-906, see entire document. | 1-15 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>12 NOVEMBER 1996 | Date of mailing of the international search report<br>26 NOV 1996 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>PHILLIP GAMBEL<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*